Abstract # LB-B18

Epithelial to Mesenchymal Transition in Human Tumor Biopsies: Quantitative, Histopathological Proof of the Existence of EMT *in vivo* by Immunofluorescence Microscopy

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Introduction

Epithelial to mesenchymal transition (EMT) in cancer, a dynamic process involving the loss of E-cadherin and gain of vimentin, is driven by changes in expression of transcription factors (Snail, Slug, Twist, and Zeb1) leading to a switch from apico-basal to front-rear polarity, increased motility, tumor cell invasion, and most likely a worse prognosis (1-4). Clinically, EMT is still widely regarded as a hypothesis-based phenomenon derived mainly from studies of human cancer cell lines *in vitro* and in mouse models (1,3). Histological evidence of tumor cells undergoing EMT in clinical biopsies is still debatable (5-7), largely due to difficulties distinguishing tumor cells with mesenchymal phenotype from neighboring mesenchymal stromal cells of the tumor microenvironment by conventional histological staining (8). β-catenin is a multifunctional protein that associates with E-cadherin at the membrane of epithelial tumor cells or, in some mesenchymal tumors, with APC/Axin/GSK3 in the cytoplasm or the nucleus after translocation (11). Using established phenotypic markers (9,10), our study shows that β-catenin is a general biomarker for demarcating (segmenting) malignant cells in tissue samples and that β-catenin segmentation enables the precise, specific and unbiased quantitation of EMT biomarkers in tumor cells while excluding surrounding stromal cells from tissue analysis. Using this method and Definiens® software-based image analysis, we demonstrate the histopathological existence of EMT in human tumor biopsies.

Methods

1. **Baseline Biopsies from Clinical Trials** (A)
   - Clinicaltrials.gov (12)
     - NCT01468922
     - NCT01748825
     - NCT01989546
     - NCT01051635

   Animal Tumor Models (B) → pazopanib or vehicle
   - Gastric Carcinomas
     - MKN45
     - SNU5

   Tumor Reference Materials and Calibrators (C)
   - Cell Lines
     - MKN45
     - GTL16
     - SKMEL28
     - SNU5
   - Xenograft Tumors
     - HT29
     - SUM149PT
     - MDA-MB-231

2. **Snap Frozen Tumor Samples**
   - 18 Ga Clinical Core Tumor Biopsies
   - Xenograft Tumor Quadrants
   - Cell Line Pellets

   SOP340507 (13)

3. **FFPE Tissue Blocks 5 um Serial Sections**
   SOP340522 (13)

4. **Processed and Stained Slides**
   - Immunohistochemistry
   - EMT Multiplex IFA

5. **Aperio Whole Slide Scanner**
   - Brightfield (H&E, Chromogenic)
   - Fluorescence (Multichannel FL)

6. **H&E Annotation by Pathologist**
   - Define evaluable tumor regions
   - Exclude regions of non-interest

7. **EMT Immunofluorescence of Adjacent Sections**
   - Define the 4 stained layers per image (DAPI, E-cadherin, β-catenin and Vimentin)
   - Extract regions of interest (ROIs) as SVS files with 4 separate layers into Definiens.
   - Each ROI has dimensions of 1.2 x 10^3 square pixel boxes (equivalent to 3.10 x 10^5 um^2 area)
   - Label each ROI or field as:
     - Pre-ROI 1, 2, 3 etc

8. **Definiens Image Analysis**
   - Segment tumor areas from stroma using β-catenin layer
   - Segment and threshold for nuclei using DAPI-stained layer
   - Threshold EMT marker layers to quantify:
     - E-Cadherin
     - Vimentin
   - Determine individual EMT marker areas per ROI; areas of overlap; total number of nuclei (or cells) per tumor ROI

9. **Calculations per Patient Sample**
   Excel, Prism, Spotfire
   - Calculate for each ROIs, per slide, per patient sample:
     - Log_{10} (V:E) -/+ SD ratio
     - Mean -/+ SD EMT marker areas/cell (um^2)

Assay Development

Development and validation of the EMT Immunofluorescence Assay

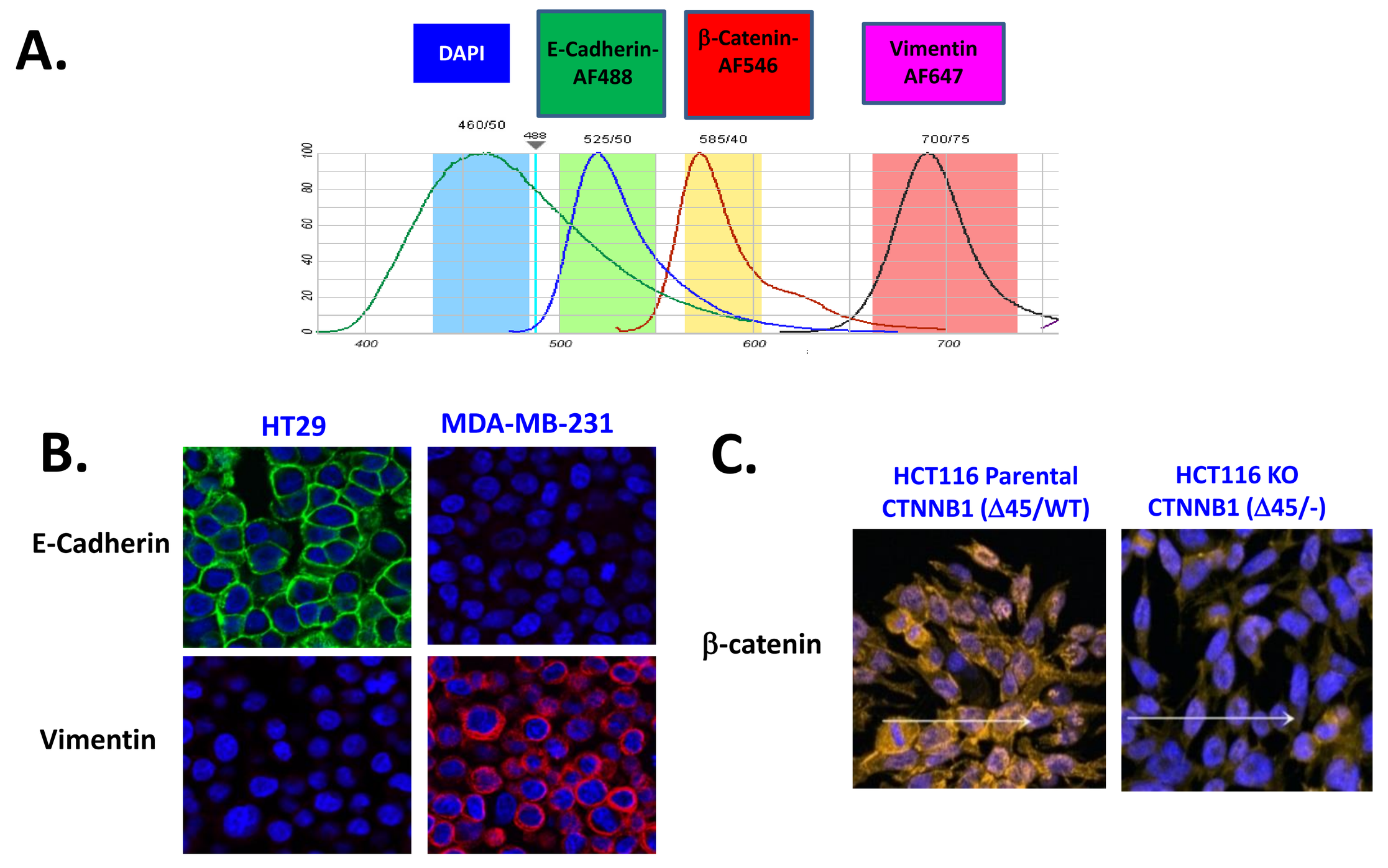
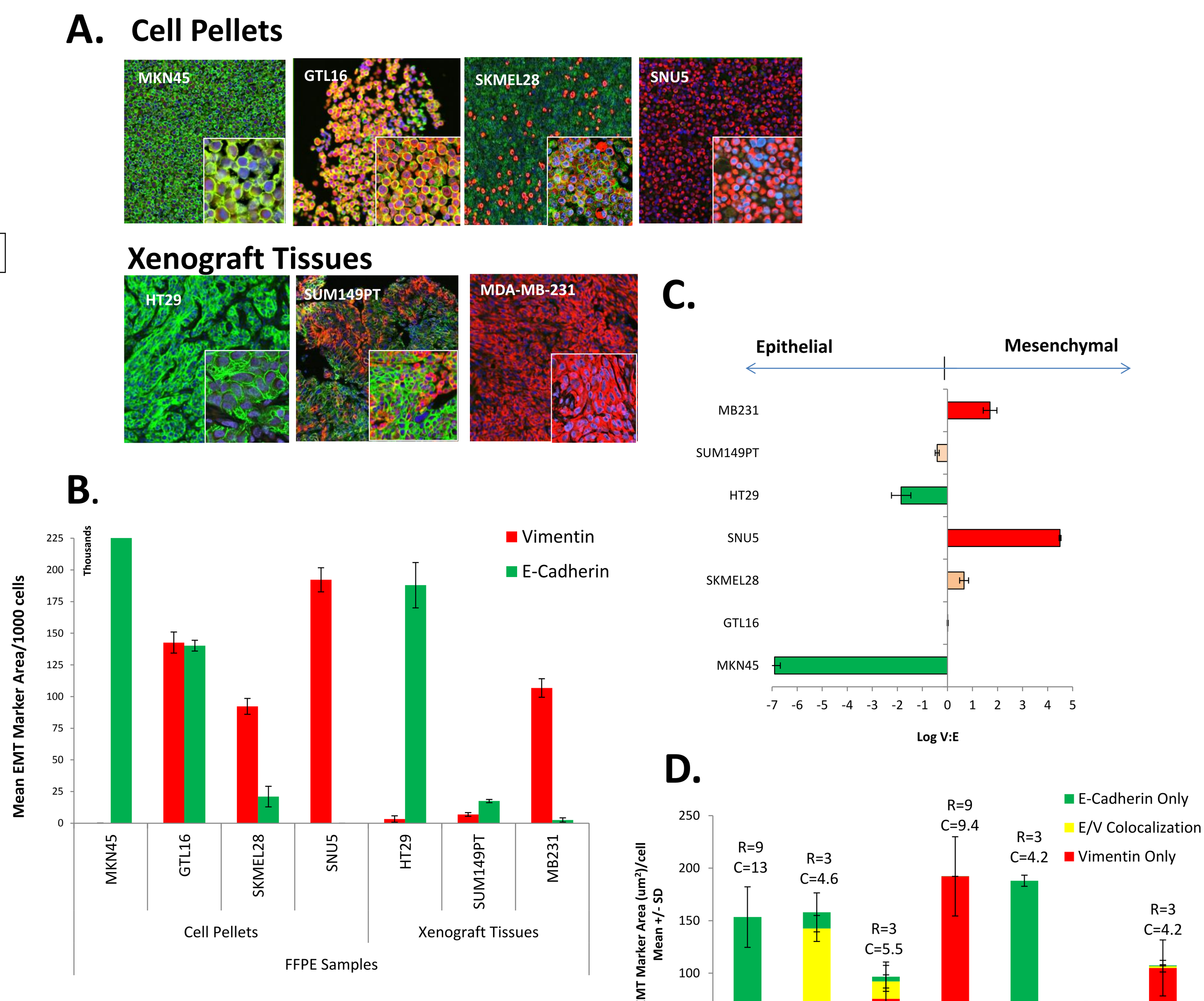


Figure 1. Development and validation of the EMT Immunofluorescence Assay

A. EMT Multiplex Immunofluorescence assay was developed using directly conjugated monoclonal antibodies to E-Cadherin (AF488), β-catenin (AF546) and Vimentin (AF647). Shown are emission fluorescence spectra for each antibody in the multiplex.
B. Validation of antibody specificity for E-cadherin and vimentin on epithelial (HT29) and mesenchymal (MDA-MB-231) tumor cell lines by immunofluorescence confocal microscopy.
C. Validation of β-catenin antibody specificity on parental HCT116 WT vs heterogeneous CTNNB1 KO HCT116 cell line by immunofluorescence confocal microscopy.

Calibrator controls and reference materials for EMT-IFA quantitative analysis



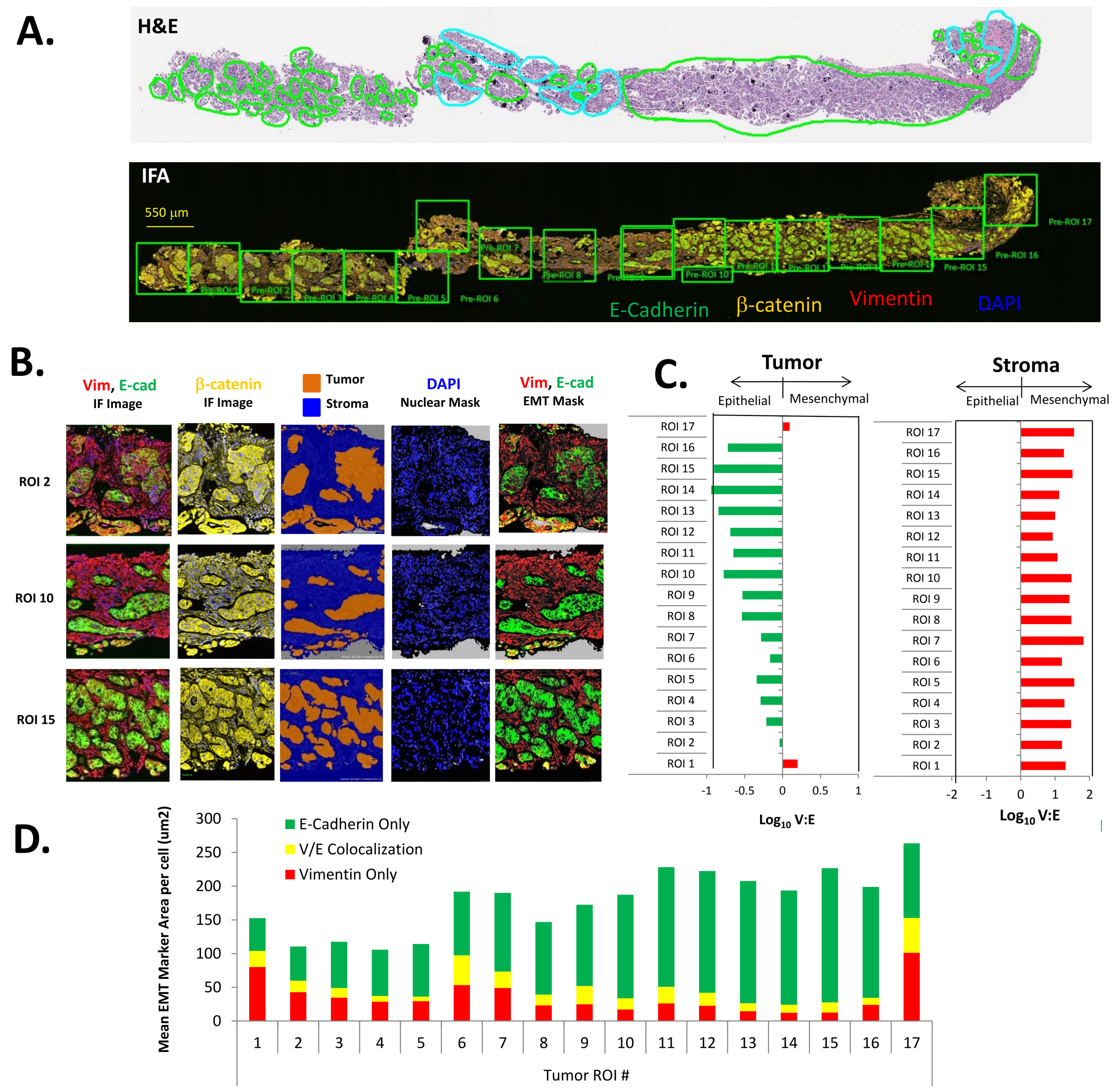
A. Cell Pellets
MKN45
GTL16
SKMEL28
SNU5
Xenograft Tissues
HT29
SUM149PT
MDA-MB-231
B.
Mean EMT Marker Area/1000 cells
Thousands
Vimentin
E-Cadherin
MKN45
GTL16
SKMEL28
SNU5
HT29
SUM149PT
MB231
Cell Pellets
Xenograft Tissues
FFPE Samples
C.
Epithelial
Mesenchymal
MB231
SUM149PT
HT29
SNU5
SKMEL28
GTL16
MKN45
Log V:E
D.
E-Cadherin Only
E/V Colocalization
Vimentin Only
Mean +/- SD

Figure 2. Development of EMT-IFA staining control calibrators and reference materials for quantitative analysis

A. FFPE cell pellet and xenograft tissues representing a spectrum of tumor phenotypes used as control calibrators and reference materials.
B. Mean EMT marker area quantitation (um2) per 1000 tumor cells for E-cadherin (green) and vimentin (red).
C. Log10 V:E for each FFPE control samples. Mean +/- SD (N=3 ROIs).
D. Mean EMT marker pixel areas (um2/cell) -/+ SD quantitative analysis per FFPE tumor sample. E+ cells (E-cadherin-only positive area, green), V+ cells (vimentin-only positive area, red) and E+V+ cells (membrane E-cadherin and cytoplasmic vimentin co-localized cellular areas, yellow). R= total number of ROIs or fields analyzed per sample. C = total number of tumor cells analyzed (x 103) .

Results

EMT IFA quantitative analysis of a FFPE human tumor biopsy section



A.
H&E
IFA
E-Cadherin
β-catenin
Vimentin
DAPI
B.
Vim, E-cad IF Image
β-catenin IF Image
Tumor
Stroma
DAPI Nuclear Mask
Vim, E-cad EMT Mask
ROI 2
ROI 10
ROI 15
C.
Tumor
Stroma
Epithelial
Mesenchymal
Log10 V:E
D.
E-Cadherin Only
V/E Colocalization
Vimentin Only
Tumor ROI #

Figure 3. EMT IFA quantitative analysis of a FFPE tumor biopsy section.

A. Pathologist-annotated H&E and image-extracted EMT-IFA stained ovarian biopsy section.
B. Definiens analysis of 3 representative image-extracted fields from EMT-IFA-stained section.
C. Log_{10} V:E ratios of EMT marker areas generated by Definiens analysis for segmented tissue regions of each ROI for the entire biopsy. Stacked bar graphs showing Mean -/+ SD of EMT marker pixel areas (um^2/cell) by individual segmented tumor ROI. E+ (E-cadherin-only positive area, green), V+ (vimentin-only positive area, red) and E+V+ (membrane E-cadherin and cytoplasmic vimentin co-localized cellular areas,yellow).

β-catenin staining can segment tumor from surrounding stromal tissues in FFPE human tumor biopsy sections

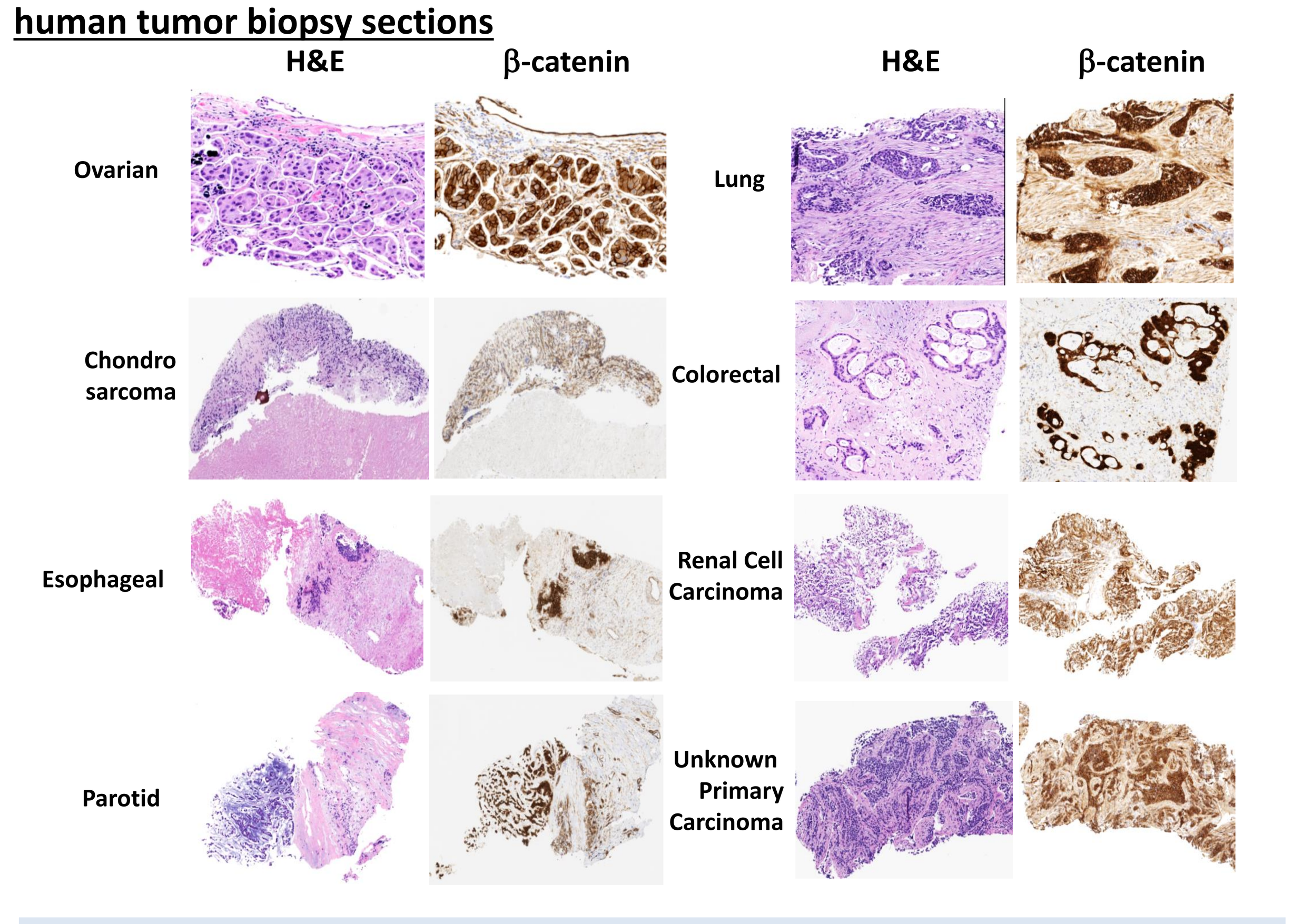


Figure 4. Comparison of H&E and β-catenin IHC staining on FFPE tumor biopsies of various histologies.
H&E (left panel) and β-catenin immunohistochemistry (IHC) (right panel) of non-adjacent tissue sections

Tumor marker staining proves that transitional cells are tumor cells

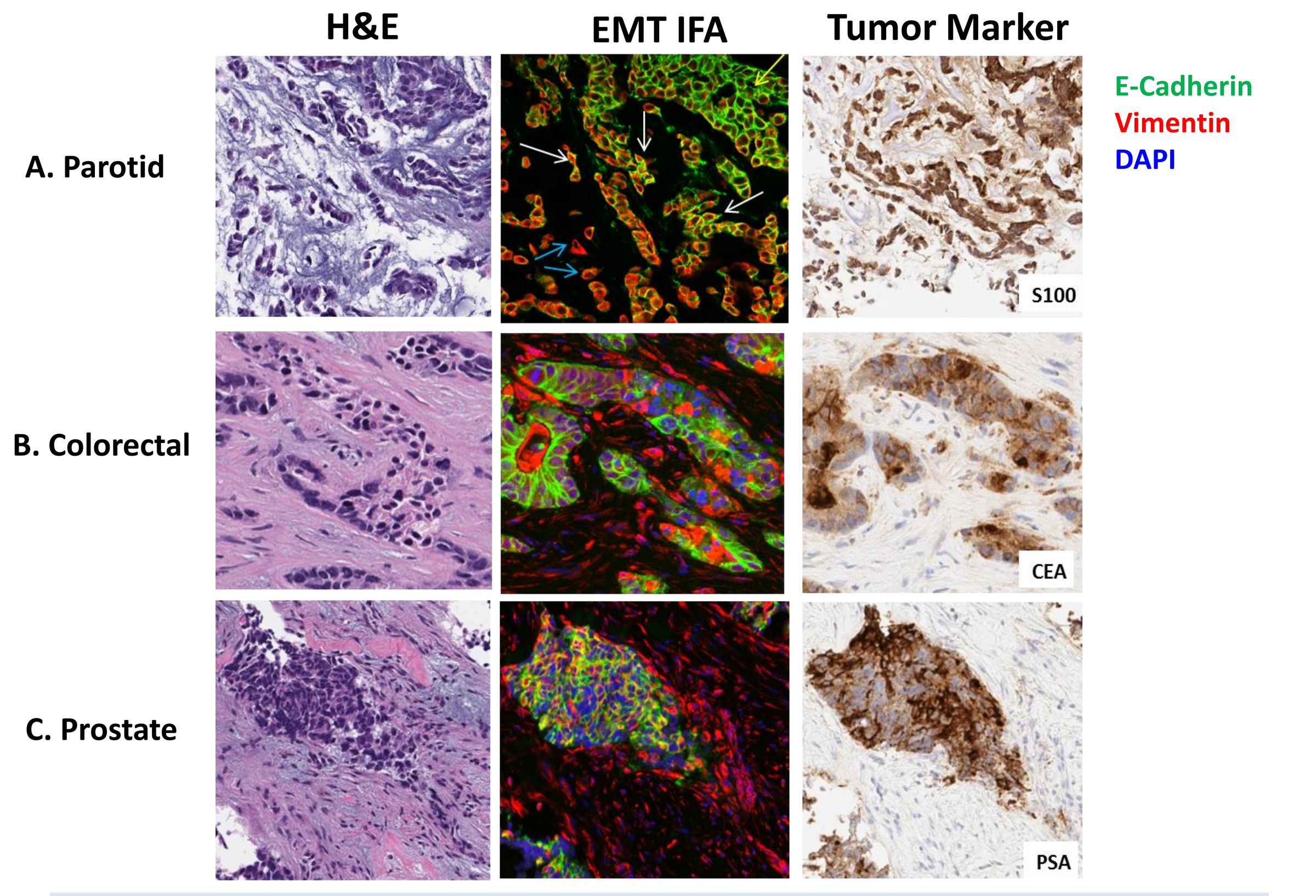
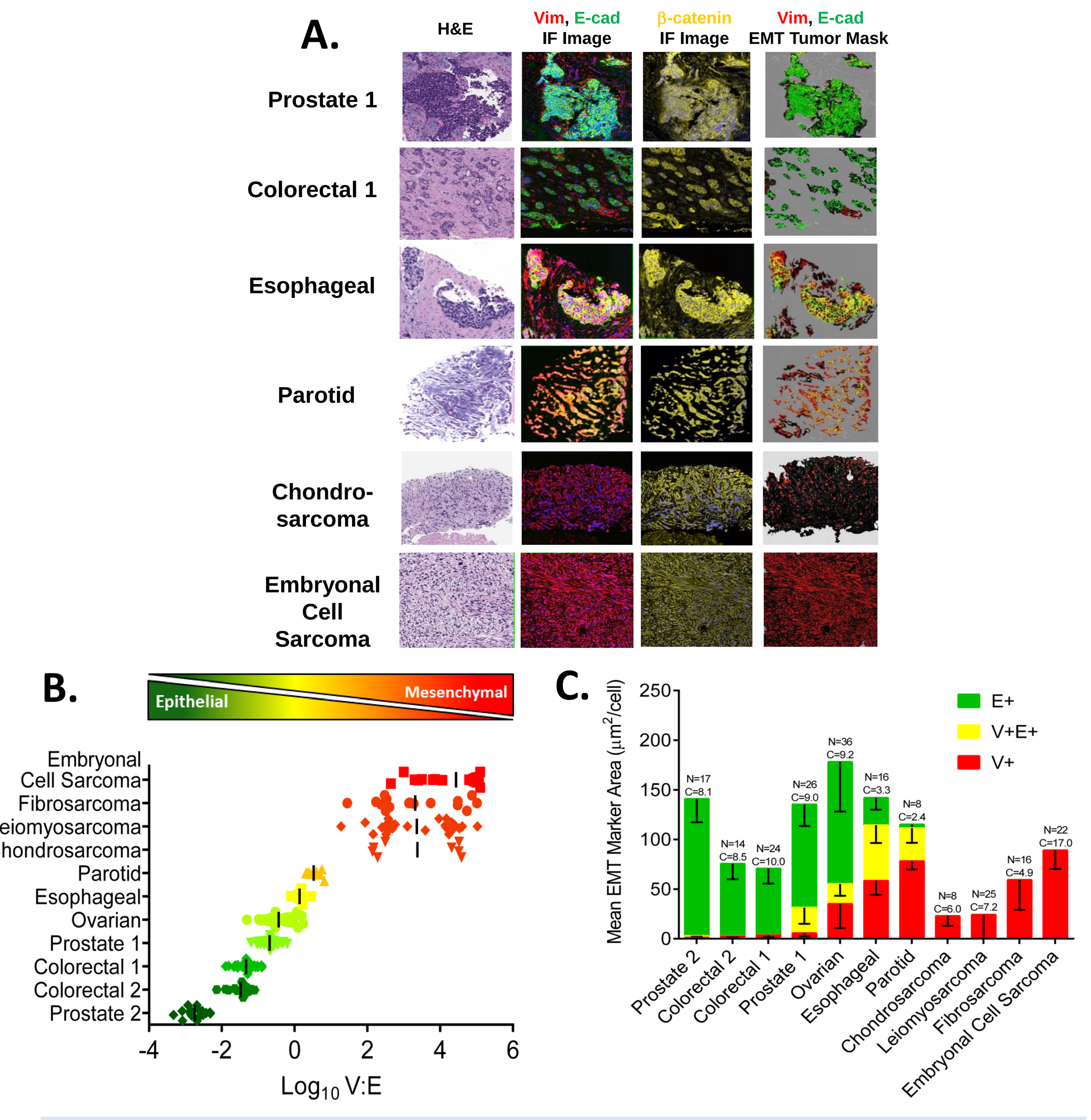


Figure 5. Representative high resolution microscopy images of tumor cells undergoing EMT *in vivo*. For each clinical case, the figure shows H&E images (left panel), overlayed E-Cadherin and Vimentin IFA staining (middle panel) and specific tumor marker staining (right panel). EMT IFA confocal image of parotid tumor (A) shows transitional cells positive for both membranous E-cadherin and cytoplasmic vimentin (white arrows), while some tumor cells are only E+ (yellow arrows) or V+ (blue arrows).

EMT IFA Analysis of various tumor histologies



A.
H&E
Vim, E-cad IF Image
β-catenin IF Image
Vim, E-cad EMT Tumor Mask
Prostate 1
Colorectal 1
Esophageal
Parotid
Chondrosarcoma
Embryonal Cell Sarcoma
B.
Epithelial
Mesenchymal
Embryonal Cell Sarcoma
Fibrosarcoma
Leiomyosarcoma
Chondrosarcoma
Parotid
Esophageal
Ovarian
Prostate 1
Colorectal 1
Colorectal 2
Prostate 2
Log10 V:E
C.
E+
V+E+
V+
Mean EMT Marker Area (um2/cell)

Figure 6. EMT IFA Analysis of various tumor histologies

A. Quantitative EMT IFA analysis of various FFPE tumor biopsies.
B. Log_{10} V:E ratios measured for individual ROIs of the biopsy section.
C. Mean EMT marker pixel areas (um^2/cell) -/+ SD for each FFPE tumor biopsy. E+ (E-cadherin-only positive area, green), V+ (vimentin-only positive area, red) and V+E+ (membrane E-cadherin and cytoplasmic vimentin co-localized cellular areas, yellow). N = total number of ROIs or fields analyzed per tumor biopsy. C = total number of tumor cells analyzed in at least 2 slide sections for each biopsy (C x10^3).

Pharmacology

Pazopanib induces EMT in preclinical xenograft models

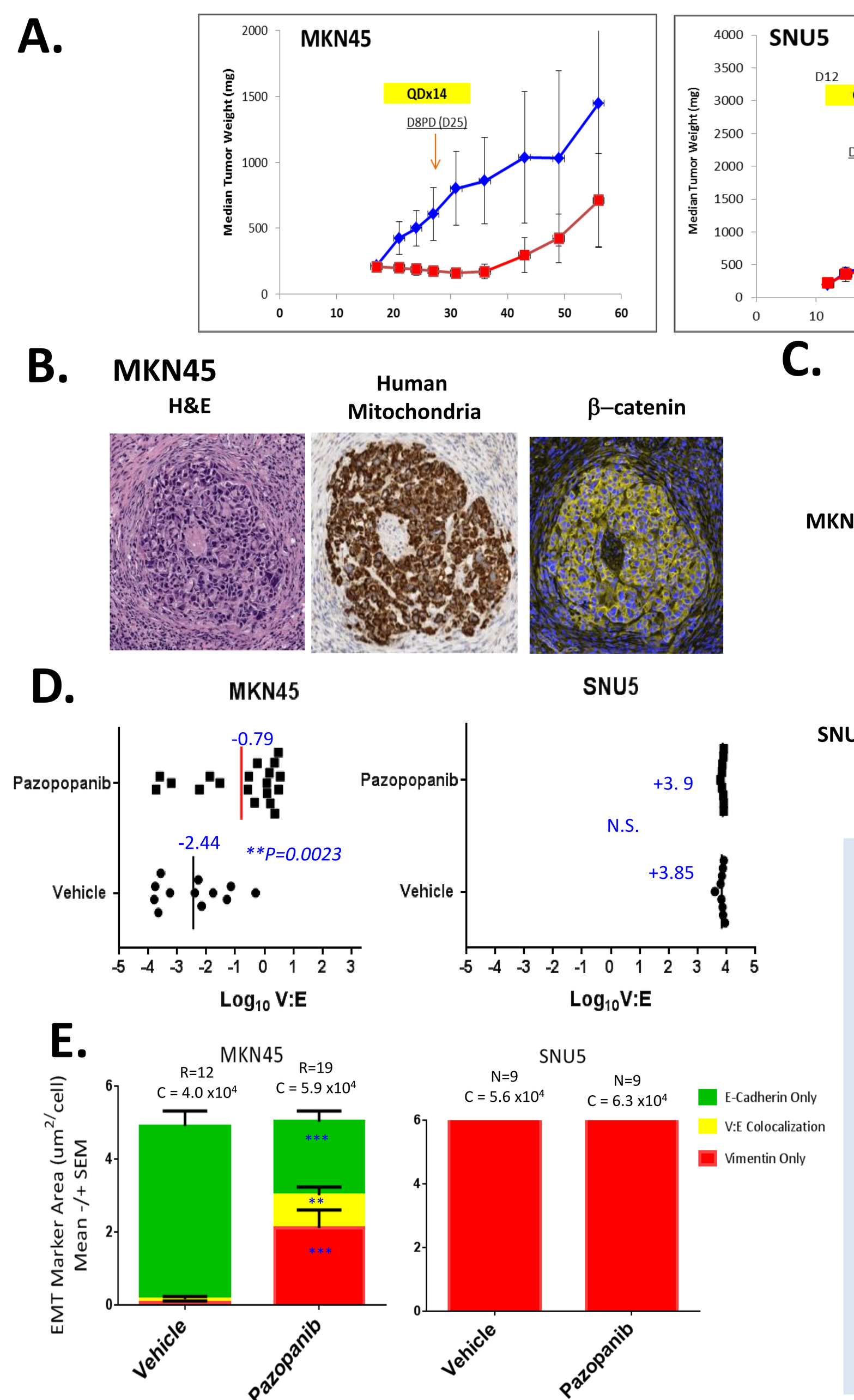


Figure 7. Pazopanib induces EMT in MKN45 xenografts

A. Growth curves showing median tumor weights -/+ SD.
B. MKN45 tumor segmentation by staining for H&E, human mitochondria and β-catenin
C. Representative tumor field images from MKN45 or SNU5 xenografts treated with either vehicle or pazopanib showing EMT marker masks for quantitative tissue analysis.
D. Log_{10} V:E ratios for ROIs of segmented tumor regions from various tumors within individual treatment groups.
E. Mean EMT marker pixel areas (um^2/cell) -/+ SEM of each xenograft tumor treated with either vehicle or pazopanib. R = total number of ROIs or fields analyzed per treatment group. C = total number of tumor cells analyzed from at least two biopsy sections.

Summary and Conclusions

- We have developed and validated a quantitative EMT-IFA assay that uses β-catenin to segment tumor cells from surrounding stromal regions coupled with a precise, quantitative and unbiased image analysis method
- Defining EMT as co-expression of the epithelial marker E-cadherin (E) and mesenchymal marker Vimentin (V) at the cellular level, a series of core needle biopsies from various tumor histologies revealed all possible phenotypes: epithelial (E+V- colorectal carcinoma), mesenchymal (E-V+ sarcomas), heterogeneous mixtures of E+V- and E-V+ subpopulations, and EMT.
- High resolution images confirmed the existence of transitional tumor cells undergoing EMT, as evidenced by co-localization of plasma membrane E-cadherin and cytoplasmic Vimentin to individual β-catenin+ tumor cells.
- Co-localization of E and V to individual tumor cells of the segmented ROIs was key for distinguishing EMT from tumor heterogeneity in which adjacent tumor cells are exclusively E+ or V+ but not E+V+.
- Pharmacological targeting of VEGFR/PDGFR/PGDFR signaling with the multi-kinase inhibitor pazopanib stimulated EMT in a preclinical xenograft model of gastric carcinoma (MKN45), as revealed by a significant increase in individual V+E+ cells (*P=0.0070*) in biopsy samples.
- This EMT-IFA has potential value for investigating EMT in the clinic and its ramifications for drug response and other clinical endpoints.

References and Acknowledgements

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12.NCT01468922: Pazopanib and ARQ 197 for Advanced Solid Tumors; NCT01748825: MK-1775 for Advanced Solid Tumors; NCT01989546: Pilot Trial of BMN 673, an Oral PARP Inhibitor, in Patients With Advanced Solid Tumors and Deleterious BRCA Mutations; NCT01051635: A Phase I Study of Indenoisoquinolines LMP400 and LMP776 in Adults With Relapsed Solid Tumors and Lymphomas. Patient samples were collected on IRB-approved protocols.
13.DCTD Standard Operating Procedures SOP340507, 340522, http://dctd.cancer.gov/ResearchResources/ResearchResources-biomarkers.htm. Animal studies were conducted according to an approved IACUC protocol.

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